CLAIMS

What is claimed is:

1. In vitro method for qualitative screening and/or quantitative determination of the functional activity of components of the Protein C anticoagulant pathway of the blood coagulation system, comprising

measuring the conversion rate of a substrate by an enzyme, the activity of which is related to the Protein C anticoagulant activity, in a blood sample of a human comprising coagulation factors and said substrate, after at least partial activation of coagulation through the intrinsic, extrinsic or common pathways and triggering coagulation by adding calcium ions; and

comparing said conversion rate with the conversion rate of a normal human blood sample determined in the same way,

said method characterized by adding additional metal ions selected from divalent metal ions and monovalent copper ions to said sample.

2. The method according to claim 1, characterized by using Mg^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Ni^{2+}, Sr^{2+}, and/or Cu^{+} ions as said additional metal ions.

3. The method according to claims 1 and 2 for the global screening for defects in the Protein C anticoagulant pathway of blood coagulation in a human, comprising:

(a) incubating a blood sample of said human comprising coagulation factors with

(1) an activator for the Protein C in said sample,

(2) a suitable coagulation activator,

(3) an exogenous synthetic substrate for either Factor Xa or thrombin comprising a photometrically measurable leaving group,

(4) calcium ions, and

(5) additional metal ions;

(b) determining the conversion rate of said exogenous substrate; and

(c) comparing said conversion rate with the conversion rate of normal human blood sample determined in the same way.

4. The method according to claims 1 and 2 for the determination of free Protein S activity in a blood sample of said human, comprising:

(a) incubating said blood sample comprising coagulation factors with:

(1) exogenous activated Protein C or exogenous Protein C together with an activator of Protein C,

(2) a suitable coagulation activator,

(3) an exogenous synthetic substrate for either Factor Xa or thrombin comprising a photometrically measurable leaving group,

(4) calcium ions, and

(5) additional metal ions;

(b) determining the conversion rate of said exogenous substrate; and

(c) comparing said conversion rate with the conversion rate of normal human blood sample determined in the same way.

5. The method according to claims 1 and 2 for the determination of Protein C activity in a blood sample of said human, comprising:

(a) incubating a blood sample of said human comprising coagulation factors with:

(1) an activator for the Protein C in said sample,

(2) a suitable coagulation activator,

(3) an exogenous synthetic substrate for either Factor Xa or thrombin comprising a photometrically measurable leaving group,

(4) calcium ions, and

(5) additional metal ions;

(b) determining the conversion rate of said exogenous substrate; and

(c) comparing said conversion rate with the conversion rate of normal human blood sample determined in the same way.

6. The method according to claims 1 and 2 for screening for Factor V mutation(s) in a blood sample of said human, comprising:

(a) incubating a blood sample of said human comprising coagulation factors with:

(1) exogenous activated Protein C, or exogenous Protein C together with an activator of Protein C, or an activator for endogenous Protein C,

(2) a suitable coagulation activator,

(3) an exogenous synthetic substrate for either Factor Xa or thrombin comprising a photometrically measurable leaving group,

(4) calcium ions, and

(5) additional metal ions;

(b) determining the conversion rate of said exogenous substrate; and

(c) comparing said conversion rate with the conversion rate of normal human blood sample determined in the same way.

7. The method according to any one of the preceding claims, characterized in that the blood sample is blood or a blood derived sample such as a blood plasma sample or a blood serum sample.

8. The method according to any one of claims 4 to 7, characterized in that said stages (1) to (5) of incubation step (a) can be performed separately and/or simultaneously.

9. The method according to any one of the preceding claims, characterized by adding said additional metal ions in the Protein C activation stage.

10. The method according to any one of the preceding claims, characterized in that said calcium ions are used at a concentration of 0.5 to 20 mmol/L, preferably 1 to 10 mmol/L, of the final assay medium.

11. The method according to any one of the preceding claims, characterized in that said Mg^{2+} ions are used in a concentration of 20 µmol/L to 10 mmol/L, preferably 100 µmol/L to 2 mmol/L and, more preferably 200 µmol/L to 1 mmol/L of the final assay medium.

12. The method according to any one of the preceding claims, characterized in that said Mn^{2+}, Zn^{2+}, Cu^{2+}, Ni^{2+}, Sr^{2+}, and/or Cu^{+} ions are used in a concentration of 1 µmol/L - 2 mmol/L, preferably 5 to 400 µmol/L and, more preferably 10 to 80 µmol/L of the final assay medium.

13. The method according to any one of the preceding claims, characterized in that the activation of Protein C in said sample precedes or occurs simultaneously with activation of coagulation.

14. The method according to any one of the preceding claims, characterized in that an activator for Protein C selected from the group

comprising Protein C activating snake venom enzymes and thrombin, if desired thrombin in combination with thrombomodulin, is used.

15. The method according to any one of the preceding claims, characterized in that a recombinant activator for Protein C is used.

16. The method according to claim 14, characterized in that a Protein C activating snake venom enzyme obtained from the Agkistrodon family of snakes, preferably from *Agkistrodon contortrix contortrix* is used.

17. The method according to claim 16, characterized in that the crude venom or the snake venom enzyme preparation Protac® C is used.

18. The method according to claim 17, characterized by using the Protein C activator Protac® C in an amount of $1x10^{-3}$ to 1 U/mL, preferably 2×10^{-3} to 0.3 U/mL in the final assay medium.

19. The method according to any one of the preceding claims, characterized by using as suitable coagulation activators for the intrinsic pathway compositions comprising

(a) phospholipid(s) and

(b) contact activators and/or activated Factors IX, XII or XI or a reagent which generates activated factors IX, XII or XI in vitro.

20. The method according to claims 18 and 19, characterized by using a contact activator selected from the group comprising ellagic acid, collagen, collagen related substances or a silica, such as micronized silica, colloidal silica and kaolin.

21. The method according to any one of the preceding claims, characterized by using native or recombinant human or non-human tissue factor (thromboplastin) from human or non-human species with or without Factor VII/VIIa, or (native or recombinant human or non-human) Factor VIIa and phospholipids as a suitable coagulation activator for the extrinsic pathway.

22. The method according to any one of claims 1, 3 to 6, 8, 19, 21 and 22, characterized by using in the at least partial activation of coagulation according to the intrinsic, extrinsic or common pathway phospholipid(s) selected from synthetic phospholipids, purified phospholipids or a mixture thereof, or crude extracts of biological sources, specifically extracts from brain, platelets, placenta, egg yolk or from soybeans.

23. The method according to any one of the preceding claims, characterized by using exogenous activated Factor X, or exogenous Factor X and an exogenous activator for Factor X, or an exogenous activator for endogenous Factor X as a suitable coagulation activator for the common pathway.

24. The method according to claim 23, characterized by using a snake venom enzyme from Russelli Viperii as an exogenous activator for Factor X.

25. The method according to any one of the preceding claims, characterized in that components of the Protein C anticoagulant pathway are added to the reaction medium to compensate for variable functional levels of such components in the sample.

26. The method according to claim 25, characterized by using components of the Protein C anticoagulant pathway selected from the

group comprising Protein C, activated Protein C, Protein S, Factor V/Factor Va, or a plasma deficient of the actual Protein C anticoagulant pathway component to be measured or a plasma deficient of all said components of the Protein C anticoagulant pathway.

27. The method according to any one of the preceding claims, characterized in that a fibrin polymerization inhibitor, such as Gly-Pro-Arg-Pro is added to the reaction medium.

28. The method according to any one of the preceding claims, characterized in that coagulation factors selected from the group comprising Factor VIII/Factor VIIIa, Factor IX, Factor X and prothrombin are added to the reaction medium.

29. The method according to any one of the preceding claims, characterized in that the coagulation factors used are selected from human or non-human sources or being produced by recombinant technology as wild-type proteins or as modified protein sequences to provide the suitable functional property.

30. The method according to any one of the preceding claims, characterized by using an exogenous synthetic substrate for either Factor Xa or thrombin in the reaction mixture.

31. The method according to claim 30, characterized in that as the exogenous synthetic substrate for either Factor Xa or thrombin a photometric substrate comprising a chromophore, a fluorophore or a luminophore as a leaving group is used.

32. The method according to claim 31, characterized in that a photometric substrate comprising a p-nitroaniline group (pNA) as a

chromophoric leaving group, a naphthylamine or coumarine derivative group as a fluorophoric leaving group, and an isoluminolamide group as a luminophoric leaving group.

33. The method according to claim 30 to 32, characterized in that as substrate for Factor Xa Benzoyl-Ile-Glu-Gly-Arg-pNA (S-2222), N-a-Z-D-Arg-Gly-Arg-pNA (S-2765), CH_3SO_2-D-Leu-Gly-Arg-pNA (CBS 31.39) or MeO-CO-D-CHG-Gly-Arg-pNA (Spectrozyme Xa) is used.

34. The method according to claim 30 to 32, characterized in that as a substrate for thrombin H-D-Phe-Pip-Arg-pNA (S-2238), pyroGlu-Pro-Arg-pNA (S-2366), H-D-Ala-Pro-Arg-pNA (S-2846), Z-D-Arg-Sarc-Arg-pNA (S-2796), AcOH*H-D-CHG-But-Arg-pNA CBS 34.47) or H-D-HHT-Ala-Arg-pNA (Spectrozyme TH) is used.

35. A kit for use in the methods according to any one of the preceding claims comprising the following components:

(a) an activator for the Protein C; or exogenous activated Protein C or exogenous Protein C together with an activator of Protein C;

(b) a suitable coagulation activator;

(c) an exogenous synthetic substrate for either Factor Xa or thrombin comprising a photometrically measurable leaving group;

(d) calcium ions; and

(e) additional metal ions;

in separate containers and/or in containers comprising mixtures of at least two of said components in aqueous solution or in lyophilized form.

36. A kit for use in the methods according to any one of the preceding claims comprising the following components:

(a) an activator for the Protein C; or exogenous activated Protein C or exogenous Protein C together with an activator of Protein C;

(b) a suitable coagulation activator;

(c) an exogenous synthetic substrate for either Factor Xa or thrombin comprising a photometrically measurable leaving group;

(d) calcium ions;

(e) additional metal ions; and

(f) coagulation factors;

in separate containers and/or in containers comprising mixtures of at least two of said components in aqueous solution or in lyophilized form.

37. A reagent for use in the methods according to any one of claims 1 to 34, characterized by comprising said additional metal ions and at least one of the following components (a) to (e):

(a) an activator for the Protein C; or exogenous activated Protein C or exogenous Protein C together with an activator of Protein C;

(b) a suitable coagulation activator;

(c) an exogenous synthetic substrate for either Factor Xa or thrombin comprising a photometrically measurable leaving group;

(d) calcium ions; and

(e) coagulation factors;

in one container in aqueous solution or in lyophilized form.

38. The reagent according to claim 37, characterized by comprising at least two of said components (a) to (e) and said additional metal ions in one container in aqueous solution or in lyophilized form.

39. The reagent according to claim 38, characterized by comprising activated Protein C, calcium ions and said additional metal ions in one container in aqueous solution or in lyophilized form.

40. The reagent according to claim 38, characterized by comprising activated Protein C, and said additional metal ions in one container in aqueous solution or in lyophilized form.

41. The reagent according to claim 37, characterized by comprising coagulation factors and said additional metal ions in one container in aqueous solution or in lyophilized form.

42. The reagent according to claim 41, characterized by additionally containing phospholipid(s).

43. The reagent according to claim 37 and 42, characterized by comprising said additional metal ions in combination with one or more of Factor V/Va, Protein C, Protein S, prothrombin, Factor VIII/VIIIa, Factor IX/IXa, Factor X/Xa, and/or thrombin in one container in aqueous solution or in lyophilized form.

44. The reagent according to claim 37, characterized by comprising said additional metal ions in combination with a Protein C activator, such as Protac®C or thrombin/thrombomodulin, in one container in aqueous solution or in lyophilized form.

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